

# **EXHIBIT 4**

15  
NEW LYMPHOKINES

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The subject matter described herein is a subject invention of NEW Grant No. IR23AI23058-03 of which the present inventor was the Principal Investigator and the Donald Guthrie Foundation for Medical Research Inc. of Guthrie Square, Sayre, PA. 18849-1669, was the Grantee.

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Lymphokines are the proteins by which the immune cells communicate with each other. Scientists produce them in sufficient quantities for therapeutic use against immunologic diseases. The present invention relates particularly to previously unknown lymphokines which are secreted from T cells in the category of helper T cells and killer (cytolytic) T cells which lymphokines were isolated and identified based on specific expression of the T cell genes using a technique identified by the present inventor in a publication (Proc. Natl. Acad. Sci. USA. 84, 2896-2900, May 1987, Immunology).

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## BACKGROUND

The immune system of humans and other species requires that white blood cells be made in the bone marrow, which white blood cells include phagocytes, lymphocytes and B cells. As presently understood, the phagocytes include macrophage cells which scavenge unwanted materials such as virus protein from the system. The lymphocytes include helper T cells and killer T cells and B cells as well as other cells, including those categorized as suppressor T cells.

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The B cells produce the antibodies. The killer T cells physically pierce the cell and the helper T cells facilitate the whole process. In any event, the immune process is facilitated by lymphokines. Interleukin 1, secreted from macrophages activate the helper T cells and raise the body temperature causing fever which enhances the activity of the immune cells. The activated helper T Cells produce Interleukin 2 and Interleukin stimulates the helper and killer T cells to grow and divide. The helper T cells also produce another lymphokine, B cell growth factor (BCGF), which causes B cells to multiply. As the number of B cells increases, the helper T cells produce another lymphokine known as the B cell differentiating factor (BCDF), which instructs some of the B cells to stop replicating and start producing antibodies. T cells also produce a lymphokine, gamma interferon (IF), which has multiple effects like Interleukin 2. Interferon helps activate killer T cells, enabling them to attack the invading organisms. Like BCGF, interferon increases the ability of the B cells to produce antibodies. Interferon also affects the macrophages to keep them at the site of the infection and help the macrophages to digest the

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5      cells they have engulfed. Gathering momentum with each kind of lymphokine signal\$ between the macrophages and the T cells, the lymphokines amplify the immune system response and the virus protein or other foreign matter on the infected cells is overwhelmed. There are many other lymphokines, maybe a hundred or more, which participate in the immune process. Many lymphokines are known and many are not.

10     Lymphokines are sometimes called intercellular peptide signals. Among scientists there is widespread use of cloned cell lines as lymphokine producers and the isolation of lymphokine mRNA has become a common technique.

15     The protocol reported in the aforesaid publication can be used by scientists to detect virtually all of the lymphokines because the method is designed to detect virtually all the mRNA expressed differentially and the mRNA sequences of the immune cells are expressed differentially as they relate to the T cells and the killer T cells even though the level of expression is low and the quantity of the secreted lymphokine protein is low. The present inventor believes that the analysis described in the above 20     identified publication can reveal biologically important molecules such as lymphokines because there are many indications that the biologically important or active molecules are coded by the most scarce messages. An example is a transforming growth factor (TGF) 25     which is present as only one of a million clones. There are many known lymphokine proteins and they include the interferons, interleukin-1,2,3,4,5,6,7, colony-stimulating factors, lymphotoxin, tumor necrosis factor and erythropoietin, as well as others.

30     Most T cell factors have been classically identified by recognizing biologic activities in assays, purifying the protein information. An alternative approach is to isolate putative T cell genes based upon specific expression and then demonstrate the function of the unknown molecule. Using the aforesaid modified differential screening procedure, the present inventor has recently cloned a series of T cell subset-specific cDNAs from cloned helper 35     T (HTL) L2 and cloned <sup>Cytolytic</sup> ~~cytolytic~~ T lymphocyte (CTL) L3.

a 40    Apparent full length cDNAs corresponding to fourteen species of the 16 initial isolates were sequenced and were found to constitute five different species. Three of the five were identical to previously reported cDNA sequences of proenkephalin, T cell replacing factor and HF gene (a serine esterase). The other, two, represented as L2G25B and 4-1BB, were novel sequences of unknown function. The open reading frames of 4-1BB and L2G25B code for 245 45    and 92 amino acids, respectively. The predicted proteins of 4-1BB and L2G25B include 22 and 23 amino acid-long putative signal sequences, respectively. The protein backbones of mature proteins encoded by 4-1BB and L2G25B are composed of 234 amino acids with

molecular weight of 25000 and 69 amino acids with molecular weight of 7880, respectively. 4-1BB contains two potential N-glycosylation sites while L2G25B has none. 4-1BB contains 23 cysteine residues in the putative mature protein.

5 The primary object is to provide the teachings identifying plural new lymphokines, including L2G25B and 4-1BB as identified herein by their gene sequence.

6 Another object of the present invention is to provide  
10 teachings of how the said new lymphokines may be used to isolate  
and identify corresponding lymphokines in related species.

15 It is still another object of the present invention to identify the gene sequence of plural new lymphokines, including L2G25B and 4-1BB.

20 Still another object of the teachings of the present invention  
15 is to teach the identification of the new lymphokines as reported  
herein.

#### ABBREVIATIONS

20 CTL, cytolytic T lymphocyte; HTL, helper T lymphocyte; LGL,  
a large granular lymphocytes; NK, natural killer cells; SDS, sodium  
~~dodecylsulfate~~  
25 ~~dodecylsulfate~~, SSC, 150 mM sodium chloride/15 mM sodium citrate,  
pH 7.0; TPA, 12-O-tetradecanoylphorbol-13-acetate. Th, helper T  
30 lymphocytes; CTL, cytolytic T lymphocytes; IL-2, interleukin 2; IL-  
3, interleukin 3; rIL-2, recombinant IL-2; CSF-GM,  
granulocyte/macrophage colony-stimulating factors; cRNA,  
complementary RNA; ss, single-stranded; ds, double-stranded; TCR,  
T-cell antigen receptor; PTA, phorbol 12-tetradecanoate 13-acetate;  
LGL, large granular lymphocytes.

#### Brief Descriptions

35 Figures 1a and 1b are flow sheets of the present inventor's approach to identifying L2 (helper T lymphocyte) specific and L3 (Cytolytic T lymphocyte) specific cDNA clones.

Figure 2a shows the nucleotide and deduced amino acid sequence of the longest reading frame of the new mouse lymphokine L2G25B thereby defining the same.

40 Figure 2b shows the optimum alignment between the new lymphokine L2G25B and a known human protein PLD78 which, as a result of the teachings herein, can also now be identified as a human lymphokine corresponding to mouse lymphokine L2G25B.

Figures 3a and 3b show the nucleotide sequence and the deduced amino acid sequence of mouse lymphokine 4-1BB.

45 Figures 4a, 4b, and 4c show an RNA blot analysis of ConA-stimulated L3 RNA with the expression being for different sizes of

lymphokine 4-1BB mRNA.

Figure 5 shows a Southern Blot analysis of mouse genomic DNA for fragments of lymphokines L2G25B and 4-1BB cDNA.

5 Figures 6a and 6b show lymphokines L2G25B and 4-1BB expressed preferentially in L2 and L3 cells only after concanavalin A stimulation.

Figures 7a, 7b, 7c and 7d show RNA Blot patterns of lymphokine L2G25B and lymphokine 4-1BB in mRNA expression TCR stimulation or IL-2 treatment.

10 Figures 8a, 8b and 8c show expression of lymphokine L2G25B mRNA and lymphokine 4-1BB mRNA in a HTL L2 and a CTL dB45 cells.

Figures 9a and 9b show the expression of lymphokine 4-1BB mRNA in concanavalin A-stimulated hybridomas PN37 and Md90 and in a unstimulated CTL CTLLAll.

15 Figures 10a, 10b, 10c and 10d show the effect of cyclosporin A on L2G25B and 4-1BB mRNA expression.

Figures 11a, 11b and 11c shows the expression of lymphokine L2G25B and 4-1BB mRNA in mouse splenocytes.

20 **MATERIALS AND METHODS**

25 Cells cloned murine CTL L3 cells, (Glasebrook, A. and Fitch, F. (1980) J. Exp. Med. 151, 876-895) are thy-1,2<sup>+</sup>, Lyt-2<sup>+</sup>, LFA-1<sup>+</sup>, LeT4<sup>-</sup> and H-2L<sup>d</sup> reactive. Cloned murine HTL L2 cells (2) are Thy-1,2<sup>+</sup>, LFA-1<sup>+</sup>, Lyt-2<sup>-</sup>, LeT4<sup>+</sup> and Mls<sup>a/d</sup> reactive.

30 Methods of isolating and maintaining the cloned helper T lymphocytes (Th), L2, and the cloned cytolytic T lymphocytes (CTL), L3, have been described in the above identified publication. To stimulate the cloned T cells, we resuspended them at 10<sup>6</sup>-10<sup>7</sup> cells per ml and cultured them with Con A (Pharmacia) at 10 ug/ml for L2 cells or 2 ug/ml for L3 cells or human recombinant IL-2 (rIL-2; Cetus) at 10<sup>2</sup>-10<sup>3</sup> units/ml. Immobilized clonotypic monoclonal antibody 384.5, which reacts with the TCR of L3 cells (Lancki, D.W., Lorber, M.I.. Loken, M.R., & Fitch, F.W. (1983) J. Exp. Med. 157, 921-935 and Moldwin, R.L., Lancki, D.W., Herold, K.C. & Fitch, F.W. (1986) J. Exp. Med. 163, 1566-1582), was used to stimulate L3 cells.

40 Mouse thymoma cells, EL4, and mouse B-cell lines, A20.2j and K46, were maintained in RPMI 1640 medium containing 5% fetal calf serum. EL4 cells were stimulated with phorbol 12-tetradecanoate 13-acetate (PTA; 10ng/ml) for up to 20 hr, monitoring the stimulation by IL-2 assay (Gillis, S., Ferm, M.M., Ou, W. & Smith, K.A. (1978) J. Immunol. 120, 2027-2032).

45 cDNA Libraries. RNAs of L2 and L3 cells that were stimulated by Con A for 14 hr, were extracted (Chirgwin, J.M., Przybyla, A.E.,

MacDonald, R.J. & Rutter, W.J. (1979) Biochemistry 18, 5294-5299) and poly(A)<sup>+</sup> mRNA was purified on an oligo(dT)-cellulose column (Aviv, H. & Leder, R. (1972) Proc. Natl. Acad. Sci USA 69,1408-1412). Double-stranded (ds) cDNA was synthesized from the poly(A)<sup>+</sup> mRNA (Land, H., Grez, M., Hauser, H., Lindenmaier, W. & Schutz, G. (1981) Nucleic Acids Res. 9, 2251-2266). The cDNA was methylated at EcoRI sites, EcoRI linkers were ligated to cDNA, and then the cDNA was enriched for molecules larger than 250,000 daltons by passage over Bio-Gel A-150m columns. The cDNAs were inserted into the EcoRI Site of gt10 bacteriophage cloning vector (Huynh, T.V., Young, R.A. & Davis, R.W. (1985) in DNA Cloning: A Practical Approach, ed. Glover, D. (IRL, Arlington, VA), Vol 1, pp. 49-78.).

cDNA Probe. Six micrograms of poly(A)<sup>+</sup> mRNA was denatured with 10 mM methylmercuric hydroxide and incubated in a buffer containing 100 mM Tris HCl at pH 8.3, 50 mM KCl, actinomycin D at 50 mg/ml, 30 mM 2-mercaptoethanol, 10, mM MgCl<sub>2</sub>, (dt<sub>12-18</sub> at 5 ug/ml, 0.5 mM each of dATP, dCTP, and dGTP, 0.01 mM dTTP, 0.001 mM [ $\alpha$ -<sup>32</sup>P]dTTP (3000 Ci mmol<sup>-1</sup>; 1 Ci = 37 GBq), and reverse transcriptase from avian myeloblastosis virus at 1000 units/ml at 46°C for 30 min. Single-stranded (ss) cDNA was freed from its template RNA by incubation in 200 mM NaOH/10 mM EDTA at 60°C for 30 min and passed over a 4-ml column of Sephadex G-100. The specific activity of the probe was usually  $\approx$ 1.6-2.0  $\times$  10<sup>8</sup> cpm/ug of cDNA.

Subtracted cDNA Probe. The ss cDNA prepared from L2 RNA was hybridized to a R<sub>0</sub>t of 1200-1500 (mol of nucleotide per liter)  $\times$  sec with poly(A)<sup>+</sup> mRNA of A20.2j in 0.41 M sodium phosphate buffer, pH 6.8, containing 0.1% <sup>SDS</sup><sub>Nabod-SQ<sub>4</sub></sub> and 1 mM EDTA, in a volume of 25-50 ul. The ss cDNA fraction was collected by chromatography through a hydroxylapatite column as recommended by the vendor (Bio-Rad). Seven percent of input cDNA was recovered in the ss fraction and used for a second round of hybridization to A20.2j poly(A)<sup>+</sup> mRNA to an equivalent R<sub>0</sub>t of 500 (mol/liter)  $\times$  sec. Approximately 93% of initial input radioactivity was recovered. Starting with 6 ug of poly(A)<sup>+</sup> mRNA, approximately 5.5  $\times$  10<sup>6</sup> cpm was obtained as a probe.

Blot DNA and RNA Hybridization. Recombinant phage DNA was prepared (Davis, R.W., Botstein, D. & Roth, J.R. (1980) Advanced Bacterial Genetics (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 106-107. and digested with EcoRI. DNA fragments were transferred to GeneScreenPlus membranes (New England Nuclear) and hybridized with ss cDNA probes (Maniatis, R., Fritsch, E.F. & Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual (Cold

Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 382-389). RNA was run on 1.2% formaldehyde denaturing agarose gel (Lehrach, H., Diamond, D., Wozney, J.M. & Boedtker, H. (1977) Biochemistry 16, 4743-4751) and transferred to GeneScreenPlus. Probes for RNA hybridization were prepared from gel-purified cDNA inserts by the random priming method (Feinberg, A.P. & Vogelstein, B. (1983) Anal. Biochem. 132, 6-13). Total cytoplasmic RNA of poly(A)<sup>+</sup> RNA were fractionated on 1.2% agarose-formaldehyde gels and transferred to GeneScreenplus (NEN, Boston, MA). Gel-purified cDNA inserts were [<sup>32</sup>P]-labeled by nick translation and used as probes. When a Northern blot of GeneScreenplus was used multiple times for hybridization, the previous probe was removed by treating the membrane in 10 mM Tris-HCl (pH7.0) and 0.2% SDS at 85°C for 1 hr.

High molecular weight DNA of mouse spleens was prepared as described previously (Grass-Bellard, M., Oudet, P. and Chambon, P. (1973) Eur. J. Biochem. 36, 32-38). Endonuclease digests of DNA were electrophoresed in 0.8T agarose gel at 4°C. The DNA was denatured, and transferred to GeneScreenPlus as described by Southern. (Southern, E. (1975) J. Mol. Biol. 98, 503-517). The blot hybridized with [<sup>32</sup>P]-labeled cDNA inserts.

L2 cells were stimulated with concanavalin A (10 ug/ml) for 14 hr, at a cell concentration of 10<sup>6</sup>-10<sup>7</sup>/ml. L3 cells were stimulated with concanavalin A (2 ug/ml) for 14 hr, at a cell concentration of 2.5 x 10<sup>6</sup>/ml. Mouse thymoma EL-4 cells (Farrar, J., Fuller-Farrar, J., Simon, P., Hilfiker, M., Stadler, B. and Farrar, W. (1980) J. Immunol. 125, 2555-2558) were stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA, 10 ng/ml) at a cell concentration of 1.0 x 10<sup>6</sup>/ml for 20 hr; stimulation was monitored by IL-2 assay (Gillis, S., Ferm, M., Ou, W. and Smith, K. (1987) J. Immunol. 120, 2027-2032.. B cell lymphoma K46 (Kim, K., Kanellopoulos-Langevin, C., Merwin, R., Sach, D. and Asofsky, R. (1979) J. Immunol. 122, 549-554), and rat Nk cell LGL (Henkart, P., Millards, P., Reynolds, C. and Henkart, M. (1984) J. Exp. Med. 160, 75-93) were not stimulated with any of above reagents.

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Figures 1a and 1b.

Fig. 1a and b are flow sheets of the present inventor's approach to identify L2-Specific and L3-specific cDNA clones. Therein: 1) the \* means that preparation of subtracted L2 cDNA probe is described in Materials and Methods; 2) the + means the probes of known sequences were prepared for cDNAs of granulocyte/macrophage colony-stimulating factors (CSF-GM), interleukin 3 (IL-3), IL-2, TCR  $\alpha$ -chain, TCR  $\beta$ -chain, c-myc, and c-fos; 3) ++ means the insert of each negative recombinant phage DNA was gel-purified and used as a probe for RNA blot hybridization ("Northern") analysis of K46,

unstimulated or PTA-stimulated EL4, large granular lymphocytes (LGL), and unstimulated or Con A-stimulated L2 and L3. The  $^{32}\text{P}$ -labeled cDNA probe, prepared from poly(A)<sup>+</sup> mRNA of A20.2j, was used to screen the library. The total cDNA probe could detect a clone corresponding to 0.02-0.05% of the test mRNA (Kwon, B.S., & Weissman, S.M. (1984) *J.Virol.* 52, 1000-1004). Of 18,000 plaques from the L2 cDNA library, 614 (3.4%) failed to hybridize to the B-cell cDNA probe. The subtracted L2 cDNA probe was hybridized to these 614 plaques and 114 (18%) gave a signal; 372 plaques gave no signal to the subtracted L2 cDNA probe or B-cell cDNA probe. Of those 372, 72 clones (19%) contained cDNA inserts. The 186 (114 + 72) clones from the L2 cDNA library were subjected to further analysis.

By similar analysis of approximately 8000 L3 cDNA clones, 150 plaques (2.0%) that failed to hybridize to  $^{32}\text{P}$ -labeled B-cell cDNA probe were selected. Instead of screening the 150 plaques with subtracted L2 cDNA probe, we digested recombinant phage DNA of each clone with EcoRI and immobilized the fragments on the filter.  $^{35}\text{S}$ -labeled B-cell cDNA probe was used to hybridize to the filters. The use of  $^{35}\text{S}$  for cDNA labeling and Southern analysis increased the sensitivity at least 5-fold. Fifty-six inserts (Fig. 1b) from L3 were identified, each of which failed to hybridize to the B-cell cDNA probe.

One Hundred and eighty-six L2 cDNA inserts and 56 L3 cDNA inserts were hybridized to cDNAs of CSF-GM, IL-3, IL-2, TCR  $\alpha$ -chain, TCR  $\beta$ -chain, c-myc, and c-fos. Twelve clones hybridized to cDNA, for IL-3, 6 to CSF-GM, 3 to IL-2, 2 to TCR  $\beta$ -chain, and 1 each to TCR  $\alpha$ -chain and c-myc (Table 1). Twenty-nine clones whose cDNA inserts were less than 50 base pairs (bp) were eliminated from further study. The blots containing 132 L2 cDNA and 54 L3 cDNA inserts were hybridized to  $^{35}\text{S}$ -labeled ss cDNA probe prepared from poly(A)<sup>+</sup> mRNA of unstimulated L3 or of unstimulated L2, respectively. Sixty-one inserts of L2 cDNA hybridized to the L3 cDNA probe and 14 inserts of L3 cDNA hybridized to the L2 cDNA probe.

Table 1. T-cell specific cDNA clones isolated from L2 and L3 cDNA library

40	Origin	Group	cDNA clone	Number of
				times isolated
45	L2	1	CSF-GM	6
		2	IL-3*	12
		3	IL-2	3
		4	TCR $\alpha$ -chain	1

		5	TCR -chain	2
		6	c-myc	1
		7	pBK791	4
		8	pBK642	1
5		9	pBK671	1
		10	pBK631	3
		11	L2G53#3	1
		12	L2G95#3	1
		13	L2G95#4	1
10		14	L2G25#4	1
		15	L2S35#3	1
			Total	39
15	L3	1	TCR B-chain	2
		2	L3G29#4	1
		3	L3G25#4	1
		4	L3G14#2	1
		5	L3G10#6	1
20		6	L3G7#1	1
		7	L3G18#3	1
		8	L3G26#1	2
			Total	10
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T-cell-specific cDNA clones were isolated from  $\approx$ 18,000 clones of L2 library and  $\approx$ 8000 clones of L3 library. After enrichment of T-cell-specific sequences, cDNA clones for CSF-GM, IL-3, IL-2, TCR  $\alpha$ -chain, TCR  $\beta$ -chain, and c-myc were detected by hybridization with the corresponding full-length cDNA provided by other laboratories. By cross-hybridization, the other clones (14 from L2 and 8 from L3) turned out to represent 16 different genes (9 from L2 and 7 from L3). Those cDNA clones representing 16 different genes were subjected to further analysis.

A partial sequence analysis revealed that the IL-3-related clones contained two different species.

The 71 (132 - 61) inserts from L2 and 40 (54 - 14) inserts from L3 were used as probes with blots of 10 ug of poly(A)<sup>+</sup> mRNA from K46, LGL (rat NK cell) (Henkart, P.A., Millard, P.J., Reynolds, C.W. & Henkart, M.P. (1984) J. Exp. Med. 160, 75-93), unstimulated or PTA-stimulated EL4, and 10 ug of total RNA from unstimulated or Con A-stimulated L2 or L3 cells.

Among these inserts, 29 ( $\approx$ 40%, 29/71) from L2 and 19 ( $\approx$ 47%, 19/40) from L3 hybridized to K46 or all lanes. Fourteen inserts ( $\approx$ 20%, 14/71) from L2 hybridized only to Con A-stimulated L2, or

both of L2 and L3 RNA. Those cDNA inserts represented nine different cDNAs. From L3, 8 (20%, 8/40) were T-cell specific, representing seven different genes; one gene was inducible by Con A in both L2 and L3, three genes were expressed constitutively and inducible by Con A only in L3 cells; and the rest were inducible by Con A in L3 cells.

A in L3 cells but not found in unstimulated L3 cells. Twenty-eight inserts ( $\approx$ 40%, 28/71) from the L2 cDNA library and 13 inserts ( $\approx$ 32%, 13/40) from the L3 library did not hybridize to any of the RNAs. Because less L2 or L3 RNA was available for blot hybridization analysis, we have not been able to eliminate the possibility that those inserts not expressed in K46, EL4, or LGL could still be expressed in L2 or L3 at a low level.

#### Screening of cDNA Library and DNA Sequencing

L2 and L3 cDNA libraries which were previously prepared were rescreened with cDNA insert of each of 14 T Cell-specific genes. Typically 10 positive clones were chosen for each species and the sizes of cDNA inserts were determined. The longest cDNA inserts were employed for nucleotide sequence analysis. DNA restriction fragments, subcloned in M13 vectors (Messing, J., Crea, R. and Seeburg, P. (1981) Nucleic Acids Res. 9,309-322), were sequenced by the dideoxy chain termination technique (Sanger, F., Nicklen, S. and Coulson, A. (1977) Proc. Natl. Acad. Sci USA 74,5463-5467) employing Sequinase (U.S. Biochemical, Cleveland, OH), with modification made to accommodate 2"-deoxyadenosine 5"- [ $\alpha$ [<sup>35</sup>S] thio]triphosphate (Biggin, M., Gison, T. and Hung, G. (1983 Proc. Natl. Acad. Sci. USA 80,3963-3965).

#### Nucleotide and Protein Sequence Comparison

Full length cDNA and predicted protein sequence were compared with the sequences in the GeneBank (NIH) DNA Sequence Library, European Molecular Biology Laboratories (EMBL) and National Biomedical Research Foundation (NBFR). Predicted protein were analyzed by Pepplot program.

Table 2 summarizes T cell cDNAs identified from 14 hr ConA-stimulated L2 and L3 cDNA libraries. Besides the cDNAs listed in the table, CSF-GM, IL-2, IL-3,  $\alpha$ -,  $\beta$ -T cell receptor and c-myc cDNAs were identified by cross-hybridization of T cell enriched cDNAs with the corresponding full-length cDNA provided by other laboratories.

TABLE 2. SUMMARY OF cDNA CLONES IDENTIFIED

	cDNA Clone*			
45	Full Length cDNA	Isolated Previously	Specificity of Expression	Identification

	4-1BB	L3G29#3, L3G25#4, L3G14#2	L2 and L3	unknown
5	L2G25B	L2G25#4, L2G95#4, L2G53#3, L2G95#3	L2 and L3	unknown (related to PLD78)
10	L2S35	L2S35#3, L2PBK671	L2 only	proenkephalin
15	8-1R	L2PBK791, L2PBK642, L2PBK631	L2 and EL-4	T cell replacing factor
20	L3G10	L3G10#6, L3G18#3	L3 only	HF gene (serine esterase)
N.D.**	L3G7#1	L3 AND EL-4	unknown	
N.D.**	L3G26#1	L3 and EL-4	unknown	

25 \* The cDNA clones were isolated independently and described as separate clones in the above identified publication May 1987 Proc. Natl. Acad. Sci. USA. 84, 2896-2900.

\*\* The full length version of the two clones was not isolated.

30 Among the 16 unidentified T cell genes, two represented proenkephalin which was identical to the sequence reported by Zurawski et al (Zurawski, G., Benedik, M., Kamb, B.J., Abrams, J.S., Zurawski, S.M. and Lee, F.D. (1986) Science 232.772-775), three were T cell replacing factor (Kinachi, T. (1986) Nature 325,70-73), and two represented T cell serine esterase gene (Gershenson, H.K. and Weissman, I.L. (1986) 232.854-858).

35 40 Four species were from different regions of cDNA represented as L2G25B( 800 bases pairs). L2G25B was homologous to a human cDNA PLD 78 915) of unknown function. Three Species (L3G29#4, L3G25#4 AND L3G14#2) were from different regions of 4-1BB (2,400 base pairs). There were no reports of sequences homologous to 4-1BB. L3G7#1 and L3G26#1 were not characterized vigorously since we could not isolate longer inserts and their expression was very low in L3.

45 In the previous studies, we also isolated 13 L3 cDNAs and whose specificity were not assigned by RNA blot analysis. One of them (l3-1) was 64% homologous to reported T cell serine esterase

(Lobe, C.G., Finlay, B.B., Paranchych, W., Paetkau, V.M. and Bleackley, R.C. (1986) Science 232, 858-861). The sequence was reported as a new member of T cell serine esterase (Kwon, B., Kestler, D., Lee, E., Wakulchik, M. and Young J. (1988) J. Exp. Med. (1988) (In press)).

Fig. 2a shows the nucleotide and deduced amino acid sequence of the longest open reading frame of L2G25B. The open reading frame codes for 92 amino acids including a putative signal sequence. The deduced sequence of the first 23 amino acid residues has characteristics of the signal peptide of secretory proteins in that it mainly contains hydrophobic amino acids and terminates with a serine residue having a small side chain (Blobel, G. and Dobberstein, B., (1975) J. Cell Biol. 67, 852-862 and Steiner, D., Quinn, P., Chan, S., Marsh, J. and Tager, H. (1980) Ann. N.Y. Acad. Sci. 34, 1-16). Therefore the mature protein is composed of 69 amino acids with molecular weight of ~7880. There is no potential N-glycosylation sites, or transmembrane-like domain. The 3<sup>rd</sup> untranslated region has characteristics of other known lymphokines (AT rich) (Shaw, G. and Kamen, R. (1986) Cell 46, 659-667).

An optimum alignment between L2G25B and PLD78 is shown in Fig. 2b. The identity of amino acids between the two proteins was approximately 80%. The evolutionary conservation of these molecules from mouse to human may indicate that they play an important role in T cell function.

Figure 2a shows the nucleotide sequence of L2G25B and the deduced amino acid sequence. The nucleotide sequence of the message strand is numbered in the 5' and 3' direction. The 5' noncoding sequence is indicated by negative numbers. Nucleotide residue 1 is the first nucleotide of the ATG initiation codon. The predicted amino acid sequence is shown below. Potential signal peptide is underlined. Consensus polyadenylation signal is boxed. Stop codon is indicated by (---).

Figure 2b shows the optimum alignment between L2G25B and PLD78-deduced amino acids. Homology search revealed that the amino acid sequence of L2G25B showed an extensive homology with a reported human sequence, PLD78 (Obara, K., Fukuda, M., Maeda, S. and Shimada, K. (1986) J. Biochem. 99, 885-894) of unknown function, whose expression is inducible by TPA in human tonsillar lymphocytes. The identity of amino acids between the two proteins was approximately 80%. \*: Identical amino acids in these proteins. +: Chemically similar amino acids found in both sequences.

The nucleotide sequence of three overlapping cDNA clones represented by 4-1BB was determined according to the strategy shown in Fig. 3a. The nucleotide sequence of 4-1BB revealed a single

long open reading frame, beginning with the ATG codon at nucleotide residues 1-3 (Fig. 3b.). This reading frame codes for a polypeptide of 256 amino acids with a molecular weight of 27,587. The assigned ATG is preceded by an in-frame termination codon TGA (nucleotide residues -12 to 9). The sequence flanking the assigned ATG (nucleotide residues -5 to 4) is a favored sequence for eukaryotic initiation sites (consensus; CCG/ACCATGG) described by Kozak (Kozak, M. (1984) *Nucleic Acids Res.* 12,857-872). In fact, 8 out of 9 consensus sequences were identical to the sequences flanking to the assigned initiation codon. The codon specifying carboxy-terminal leucine is followed by the translational termination codon TGA (nucleotide residues 659-771). 4-1BB contains 1434 nucleotides of 3'-untranslated region which did not extend as far as polyadenylation signal nor the poly (A)<sup>+</sup> tail.

Figure 3 shows the nucleotide sequence and the deduced amino acid sequence of 4-1BB. The nucleotides of the message strand are numbered in the 5' to 3' direction and numbers are shown on both sides of the sequence. Nucleotide residue 1 is the A of the initiation codon ATG, and the nucleotides on the 5' side of residue 1 are indicated by negative numbers. The predicted amino acid sequence is shown below the nucleotide sequence. Putative signal peptide is underlined. The potential asparagine-linked glycosylation sites are underlined. Potential polyadenylation signal is boxed. Stop codon is indicated by (---). Cysteine residues are highlighted by (·). An unusual feature of 4-1BB sequence is that there is a potential polyadenylation signal of AATAAA at nucleotides 1158-1163 (Fig. 3b boxed). We believe that this signal is functional because this gene produces at least two different sizes of mRNA. We believe that this signal of AATAAA at nucleotides 1158-1163 (Fig. 3b boxed). We believe that this signal is functional because this gene produces at least two different sizes of mRNA. Fig. 4a and b shows RNS blot analysis of ConA-stimulated L3 RNA. When the blot was hybridized to L3G25#4 probe which contained sequences of 3' side to the polyadenylation signal (nucleotides 1284-1557). The probe detected one RNA species of approximately 2.4 kb. When the same blot was hybridized to L3G14#2 probe which contained sequences of 5' side to the first polyadenylation signal (nucleotides 661-855), the probe detected two mRNA species of approximately 1.5 kb and 2.4 kb.

Figure 4 shows the expression of two different sizes of 4-1BB mRNA. Ten micrograms of poly(A)<sup>+</sup> mRNA from mouse B cell line (K46), TPA-stimulated EL-4 (EL-4 TPA) and rat NK cell line (LGL), and ten micrograms of total RNA from unstimulated L3 (L3) and concanavalin A-stimulated L3 (L3 ConA) were fractionated on a 1.4% formaldehyde agarose gel, transferred to GeneScreenPlus and hybridized to [<sup>32</sup>P]-labeled L3G25#4 (a), L3G14#2 (b) and L3G20#3

(c) sequentially. L3G25#4 and L3G14#2 represent cDNA fragments of the 3' side and 5' side to boxed AATAAA sequence, respectively.

L3G20#3 is an anonymous cDNA from L3 cDNA library and is used to show that each lane of the blot contains a similar amount of RNA. Positions of 28S and 18S rRNA markers are each indicated. Arrows indicate the specific hybridization signal.

The deduced sequence of the first 22 amino acids of 4-1BB has characteristics of the signal peptide of secretory and membrane-associated protein (Blobel, G. and Dobberstein, B., (1975) *J. Cell Biol.* 67, 852-862), which mainly contains hydrophobic amino acids. We putatively assigned the first 22 amino acids as a signal peptide. A possible cleavage site of the signal peptide is after glycine residue at alanine (Fig. 3b). Gly-ala at amino acid\positions 22 and 23 is one of the favorable signal peptidase cleavage sites(\*). Thus the protein backbone of processed 4-1BB protein is composed of 234 amino acids with a molecular weight of 25,000. We found two potential asparagine-linked glycosylation signals (22,23) at amino acid positions 129 and 138 as underlined in Fig. 3b. The predicted 4-1BB protein contains unusually large numbers of cysteines. There are 23 cysteine residues in the putative mature protein as dotted in Fig. 3a.

There is a stretch of 26 amino acids that constitutes hydrophobic domain toward the carboxy terminus of the protein (amino acids at positions 182-211). Whether this region serves as a membrane-spanning domain is not known. This region is followed by the 45 amino acids which constitute a hydrophilic region.

#### Southern Blot Analysis.

As shown in Fig. 5, fragments of L2G25B and 4-1BB cDNA each detect single restriction fragment of approximately 15kb and 18kb in both C57BL/6 and BALB/c, DNA, respectively. The data indicate that the genes encoding the two molecules exist as a single copy in C57BL/6 and BALB/c mice. Figure 5 shows a Southern Blot analysis of mouse genomic DNA. Genomic DNA from C57BL/6 (lanes 1,3) and BALB/c (lanes 2,4) was digested with EcoRI restriction enzyme, fractionated on a 0.8% agarose gel, transferred to Gene-Screenplus and hybridized to [<sup>32</sup>P] labelled L2G25B (lanes 1,2) and 4-1BB (lanes 3,4).

The protocol developed by the present inventor and reported and published as identified hereinabove for a modified differential screening of a cDNA library by which one can detect a broad representation of the mRNA expressed differentially in two different cell types, was applied to the systematic analysis of HTL and CTL gene expression and allowed us to isolate T cell subset specific genes. This approach offers an alternative to the classical protein purification for identifying molecules and genes.

Advantages of this method are: 1) The approach identifies the existence of molecules which otherwise may be difficult or impossible to recognize or isolate; 2) Even molecules which exist at a low level in the natural source can be produced in quantity by recombinant DNA technologies and in turn provide enough protein to permit study of function and possibly clinical applications; and 3) It is a straightforward method for identifying mutations of the gene using the nucleic acid probe. As an illustration of the usefulness of this approach, the genes for T-cell antigen receptors and X-linked immunodeficiency (xid) genes were cloned and characterized in this fashion (Hedrick, S., Nielsen, E., Kavaler, J., Cohen, D. and Davis, M. (1984) *Nature*. 308,153-158 and Cohen, D., Steinberg, A., Paul, W. and Davis, M. (1985) *Nature* 314,37-374). This approach in our hands has already been prove to be useful in isolating known as well as previously unrecognized T-cell mediators.

Using the same concanavalin A stimulated L2 cells, Prystowsky et al (Prystowsky, M., Ely, J., Beller, D., Eisenber, L., Goldman, J., Goldman, M., Goldwasser, E., Ihle, J., Quintans, J., Remold, M., Vogel, S. and Fitch, F. (1982) *J. Immunol.* 129,2337-2344) identified 10 different lymphokine activities from culture supernatants; they include IL-2, IL-3 BCSF, CSF, IFN- $\gamma$  and five unidentified factors which affect macrophage activities. In the course of the studies we isolated and identified cDNAs for IL-2, IL-3, CSF, T cell replacing factor and proenkephalins from our concanavalin A-stimulated L2 and cDNA library (2 and unpublished observations). Therefore, L2G25B AND 4-1BB might represent the novel soluble mediators of Prystowsky et al which affect macrophage activities.

By applying a modified differential screening of L2 and L3 cDNA library, two novel T cell genes were isolated. Correlation of these T cell molecules with functional activities is important.

Following is the evidence supporting that L2G25B and 4-1BB code for lymphokines.

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T-cell-specific expression of L2G25B and 4-1BB.

L2G25B was isolated from an L2 cDNA library, and 4-1BB was isolated from and L3 cDNA library by the aforesaid modified differential screening (5). As shown in Fig. 6a and 6b, L2G25B and 4-1BB were expressed preferentially in L2 and L3 cells only after concanavalin A stimulation. The sizes of transcripts were approximately 800 bases for L2G25B and 2400 bases for 4-1BB. The abundance of the two transcripts was 5 ~10 fold higher in L2 cells than in L3 cells. The two transcripts were not detectable in K46 B cells, EL-4 thymoma cells or rat large granular lymphocytes. L2G25B mRNA was consistently more abundant than 4-1BB mRNA. Figure

6 shows T cell-specific expression of L2G25B and 4-1BB mRNA. Poly (A)<sup>+</sup> mRNA was prepared from mouse B cell line (K46), unstimulated EL-4 (EL-4), TPA-stimulate EL-4 (EL-4 TPA) and rat NK cell line (LGL), and total RNA was prepared from unstimulated L2 (L2), concanavalin A-stimulated L2 (L2 ConA), unstimulated L3 (L3) and concanavalin A-stimulated L3 (L3 ConA). Ten micrograms of total RNA or ten micrograms of poly(A)<sup>+</sup> RNA was fractionated on a formaldehyde/agarose gel, transferred to GeneScreenplus and hybridized to [<sup>32</sup>P]-labelled L2G25B(a) and 4-1BB(b) sequentially. Positions of 28S and 18S rRNA markers are each indicated. An arrow indicates the specific hybridization signal.

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L2G25B and 4-1BB mRNA were inducible by TCR stimulation, but not by IL-2 stimulation.

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The inducibility of the two cDNA clones was tested after L3 TCR stimulation by clonotypic antiTCR mAb, 384.5, or IL-2. As shown in Fig 7a and 7b, the expression of the two cDNA was inducible by TCR stimulation but not by IL-2 stimulation in L3 Cells. L2G25B mRNA was detectable at 0.5 hr after TCR stimulation, peaked at 6 hr, and decreased thereafter until at least 24 hr. 4-1BB mRNA was detectable at a very low level in unstimulated L3 cells in this experiment. The induction of 4-1BB mRNA occurred approximately 6 hr after TCR stimulation and remained level until 24 hr.

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Fig 7c shows the kinetics of IFN- $\gamma$  mRNA expression in the same RNA blot as used in Fig 7a and 7b. IFN- $\gamma$  mRNA was detectable at 0.5 hr after TCR stimulation, peaked at 12 hr and declined slightly until 24 hr. There was a low level of IFN- $\gamma$  mRNA in unstimulated L3 cells. When we compared the peak level of L2G25B and 4-1BB mRNA with that of IFN- $\gamma$  mRNA, IFN- $\gamma$  mRNA was at least 20 fold higher than that of L2G25B mRNA and a least 50 fold higher than that of 4-1BB mRNA. Fig 7d demonstrates that all six lanes contained almost identical amounts of RNA. The probe was a serine protease cDNA (L3G10#6) isolated from L3 cells. In summary, the pattern of the two cDNA expression was similar to that of IFN- $\gamma$  expression. Figure 7 shows patterns of L2G25B and 4-1BB mRNA expression after TCR stimulation or IL-2 treatment. L3 cells were stimulated with clonotypic antiTCR mAb 384.5 for 0, 1/2, 6, 12 or 24 hr or with rIL-2 for 6 hr. Ten ug of total RNA was fractionated on a formaldehyde/agarose gel, transferred to GeneScreenplus and hybridized to [<sup>32</sup>P]-labeled L2G25B(a) 4-1BB(b), IFN- $\gamma$  (c) and L3G10#6(d) cDNA. L3G10#6 is a serine protease cDNA isolated from L3 cell cDNA library, which is identical to HF gene (Gershenson, H. and Weissman, I., Science. (1986) 232:854). L3G10#6 is used to show that each lane contains almost equal amounts of RNA. Positions of 28S and 18S rRNA markers are each indicated. An arrow

indicates the specific hybridization signal.

L2G25B and 4-1BB mRNA are inducible by TCR stimulation in other cloned HTL, CTL and hybridomas.

As shown in Fig 8a and 8b, L2G25B and 4-1BB mRNA are also inducible in HTL L2 and CTL dB45 after TCR stimulation with antiTCR mAb F23.1. The mRNA level for the two cDNA was also much lower than that of IFN- $\gamma$  in L2 and dB45 cells (Fig 8c). L2 cells show the highest level of expression of the three cell clones. Figure 8 shows expression of L2G25B and 4-1BB mRNA in HTL L2 and a CTL dB45 cells. HTL L2 and CTL dB45 cells were stimulated with antiTCR mAb F23.1 for 6 hr. L3 cells were stimulated with anti TCR mAb 384.5 for 6 hr. Ten ug of total RNA from unstimulated L3 (lane a) and stimulated L3 (lane 2), unstimulated dB45 (lane 3), stimulated dB45 (lane 4), unstimulated L2 (lane 5) and stimulated L2 (lane 6) was fractionated on formaldehyde/agarose denaturing gel, transferred to GeneScreenplus and hybridized to [ $^{32}$ P] labeled L2G25B(a), 4-1BB(b), and IFN- $\gamma$ (c) cDNA. A fraction of RNA in each lane was degraded and detected as RNAs in lower molecular sizes.

We also found that 4-1BB mRNA was inducible by concanavalin A in two cytotoxic hybridomas, PN37 and Md90 (Fig 9a) and detectable in unstimulated CTLLAll CTL (Fig 9b) clones.

Figure 9 shows Expression 4-1BB mRNA in concanavalin A-stimulated hybridomas PN37 and Md90, and in an unstimulated CTL CTLLAll.

- a. BW5147, PN37 and Md90 cells were stimulated with concanavalin A for 4 hr. Ten ug of poly(A) $^+$  mRNA from unstimulated and stimulated each of these cells was fractionated, transferred to nitrocellulose filter and probed with [ $^{32}$ P]-labelled 4-1BB cDNA.
- b. Ten ug of poly(A) $^+$  mRNA from mouse melanoma cells (melanocyte) and ten ug of total RNA from unstimulated L2 (L2), CTLLAll (All) and L3 (L3) cells was fractionated, transferred to Gene-Screenplus and hybridized to [ $^{32}$ P] 4-1BB cDNA.

Effects of cyclosporin A on L2G25B and 4-1BB transcription.  
To test the possibility that the two cDNAs represent two different soluble extracellular mediators, we next examined the effect of cyclosporin A on RNA expression. Cyclosporin A inhibits mitogen or antigen-induced T-cell proliferation (Morris, P., Transplantation. (1981) 32:349; Orosz, C., Fidelus, R., Roopenian, D., Widmer, M., Ferguson R. and Bach, F., (1982) J. Immunol. 129:1865 and Hess, A., Tutschka, P., Pu, Z. and Santos, G., J. Immunol. (1982) 128:360). It has also been shown to block the induction of expression of several lymphokine genes including IL-2 and IFN- $\gamma$  (Kronke, M., Leonard, W., Depper, J., Arya, S. Wong-

Stahl, F., Gallo, R., Waldmann, T. and Greene, W., Proc. Natl. Acad. Sci. USA (1984) 81:5214; Elliott, J., Lin, Y., Mizel, R., Bleackley, R., Harnish, D. and Paetkau, V., Science. (1984) 226:1439 and Granelli-Piperno, A., Inaba, K. and Steinman, R., J. Exp. Med. (1984) 160:1792). The inhibition of lymphokine production occurs at a pretranslational level (Wiskocil, R., Weiss, A., Imboden J., Kamin-Lewis, R. and Stobo, J., J. Immunol. (1985) 134:1599). In contrast cyclosporin A appears to have no effect on the inducible expression of c-fos and IL-2 receptor genes in T cells. As shown in Fig 10a and 10b, cyclosporin A inhibited the induced accumulation of L2G25B and 4-1BB mRNA. The same findings were seen with IFN- $\gamma$  (Fig 10c). A low level of expression of L2G25B mRNA was seen in TPA-stimulated EL-4 cells in this experiment. Figure 9d shows that cyclosporin A had minimal or no effect on the level of serine protease (probe, L3G10#6) mRNA and shows that the three lanes contained almost equal amounts of RNA (EL-4 or K46 cells did not express L3G10#6 mRNA). This data strongly suggest that L2G25B and 4-1BB expression may show some of the same activation requirements as other known lymphokines.

Figure 10 shows the effect of cyclosporin A on L2G25B and 4-1BB mRNA expression. L3 cells were stimulated with concanavalin A, concanavalin plus cyclosporin A or concanavalin 1A plus actinomycin D. Ten ug of total RNA from unstimulated L3(L3), concanavalin A-stimulated L3 (L3 ConA), concanavalin A plus cyclosporin A-treated L3 (L3 ConA + CsA) and concanavalin A plus actinomycin D-treated L3 (L3 ConA + ActD) cells and ten ug of poly(A)<sup>+</sup> mRNA from K46 (K46) and TPA-stimulated EL-4 cells (EL-4) were fractionated, transferred to GeneScreenplus membrane and hybridized to [<sup>32</sup>P] labelled L2G25B(a), 4-1BB(b), IFN- $\gamma$ (c) and L3G10#6(d) cDNA. Cyclosporin A treatment did not alter the level of L3G10#6 mRNA but almost completely abrogated the induced expression of other 4 mRNA species. An arrow indicates a specific hybridization signal. X

L2G25B and 4-1BB mRNA were inducible in normal mouse spleen cells.  
To find out if the expression of these genes were not unique to certain cloned T cells or hybridoma cells, splenocytes from C57BL/6, and BALB/c mice were stimulated with concanavalin A and tested for mRNA expression. As shown in Fig 11a and 11b the two mRNA were detectable after concanavalin A stimulation in C57BL/6 and BALB/c mouse splenocytes. They were also inducible in Swiss Webster mouse splenocytes (data not shown). As shown in Fig. 10c IFN- $\gamma$  mRNA was detectable in concanavalin A-stimulated BALB/c splenocytes (for unknown reasons IFN- $\gamma$  mRNA was not detectable in concanavalin A-stimulated C57BL/6 splenocytes in this experiment). RNA preparations for Figure c were different from those for Figures

a and b. These data suggest that these molecules may be induced in normal mouse spleen cells by appropriate stimuli as in the cloned T cells.

5           Figure 11 shows the expression of L2G25B and 4-1BB mRNA in mouse splenocytes. Splenocytes were obtained from C57BL/6 and BALB/c mice and stimulated with concanavalin A for 14 hr. Ten ug of total RNA from unstimulated BALB/c (lane 1) and stimulated BALB/c (lane 2), unstimulated C57BL/6 (lane 3) and stimulated C57BL/6 (lane 4) splenocytes was fractionated, transferred to Gene Screen plus end hybridized to [<sup>32</sup>P]-labelled L2G25B(a), 4-1BB(b) and IFN- $\gamma$  (c) cDNA. A portion of L3G29 cDNA (approximately 200 pairs in the middle of the molecule) consistently detects an additional RNA species of approximately 1500 bases. The additional hybridization signal is seen in figure 11b.

10           L2G25B and 4-1BB share properties which suggest that they encode soluble T cell mediators. The properties are; 1) the mRNA of the two is preferentially expressed in T cells; 2) The mRNAs of the two genes are present in undetectable amount in T cells until induced by concanavalin A, or by TCR stimulation; 3) The small size of the mRNA of L2G25 is consistent with features of several analyzed lymphokine cDNAs such as interleukins 2,3 and 5; 4) The patterns of expression are very similar to that of the lymphokine IFN- $\gamma$ ; 5) Both have a potential signal sequence and an AT rich 3' untranslated region consistent with a lymphokine gene (Shaw, G. and Kamen, R., Cell (1986) 46:659); and 6) Cyclosporin A inhibits the induced mRNA expression corresponding to the two cDNAs. Using the same concanavalin A stimulated L2 cells, Prystowsky et al (Prystowsky, M., Ely, J., Beller, D. Eisenberg, L., Goldman,J., Goldman, M., Goldwasser, E., Ihle, J., Quintans, J., Remold, M., Vogel, S. and Fitch, F., J. Immunol. (1982) 129:2337) identified 10 different lymphokine activities from culture supernatants; they include IL-2, IL-3, BCSF, CSF, T cell replacing factor and proenkephalins from our concanavalin A-stimulated L2 cDNA library (5 and unpublished observation). Therefore, L2G25B and 4-1BB might represent the novel soluble mediators of Prystowsky et al which affect macrophage activities.

Isolation of human lymphokines homologous to L2G25B and 4-1BB.

40           L2G25B and 4-1BB cDNA may be used as probes to isolate human lymphokines homologous to these type clones. Each cDNA will be radio-labeled and hybridized to human genomic DNA blot under various stringency and washing conditions using standard laboratory techniques known to those skilled in the art.

45           The species difference in nucleotide sequences between human and mouse will determine the degree of homology by clone hybridization experiments. On the determination of the optimal

hybridization and washing conditions under which the probes detect a signal in the human genomic DNA blot, then a human genomic library in lambda vector may be screened with radio labeled L2G25B and 4-1BB. The hybridizing human clones may then be isolated and the nucleotide sequences determined.

The genomic human clone corresponding to mouse clone L2G25 and 4-1BB may then be used as a probe to survey human T cells which express mRNA by RNA blot analysis. When we identify the human T cells which express the RNA homologous to L2G25B and 4-1BB, the RNA may then be used to construct a cDNA library. Then the cDNA library may be screened with the human genomic clone corresponding to L2G25B and 4-1BB and isolate the human cDNA clones corresponding to the L2G25B and 4-1BB.

Plasmid p4-1BB may be used to grow the lymphokine 4-1BB. Plasmid pL2G25B may be used to grow the lymphokine L2G25B. To do so: one must insert the cDNA of L2G25B (for example) into an appropriate prokaryotic or a eukaryotic expression vector such as a Bovine Papilloma virus expression vector; and transfecting that expression vector into mouse fibroblasts; and grow the then transfected mouse fibroblasts in an appropriate culture media; and then purifying the lymphokine protein from the culture media. The same approach can be used to grow the lymphokine 4-1BB.

cDNA in the form of plasmid p4-1BB in Ecoli NM 522 has been deposited at the American Type Culture Collection under ATC No: 67825 and will be available after this Patent Application issues.

cDNA in the form of plasmid pL2G25B in Ecoli NM 522 has been deposited at the American Type Culture Collection under ATC No: 67826 and will be available after this Patent Application issues.

The foregoing description has been directed to particular embodiments of the invention in accordance with the requirements of the Patent Statutes for the purposes of illustration and explanation. It will be apparent, however, to those skilled in this art that many modifications and changes will be possible without departure from the scope and spirit of the invention. It is intended that the following claims be interpreted to embrace all such modifications.

I claim:

*Sabita*

- 40 1. A cDNA gene encoded for a lymphokine 4-1BB.
2. A cDNA gene encoding a lymphokine 4-1BB for comprising a nucleotide sequence as shown in Figures 3a and 3b.
3. A plasmid comprising a cDNA replicon identified as p4-1BB deposited at the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATC No: 67825.
- 45 4. A cDNA gene encoded for a lymphokine L2G25B.

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5. A cDNA gene encoding a lymphokine L2G25B comprising a nucleotide sequence as shown in Figure 2a.

5

6. A plasmid comprising a cDNA replicon identified as pL2G25B deposited at the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATC No:67826.

10

7. The new use of a known human protein PLD78 where said use is as a human lymphokine.

*Sabu A.B.*

8. The mouse cDNA L2G25B used as a probe to isolate the corresponding human lymphokine.

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9. The mouse cDNA 4-1BB used as a probe to isolate the corresponding human lymphokine.

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10. The cDNA of a human lymphokine corresponding to the mouse cDNA L2G25B isolated from a human source using mouse cDNA L2G25B as a probe.

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11. The cDNA of a human lymphokine corresponding to the mouse cDNA 4-1BB isolated from a human source using mouse cDNA 4-1BB as a probe.

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12. The lymphokine L2G25B produced by  
a) inserting the cDNA of L2G25B into an appropriate expression vector.  
b) transfecting said expression vector into mouse fibroblasts.  
c) growing said thus transfected mouse fibroblasts in appropriate culture media and  
d) purifying the lymphokine protein from the said culture media.

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13. The lymphokine 4-1BB produced by  
a) inserting the cDNA of L2G25B in as appropriate expression vector  
b) transfecting said expression vector into mouse fibroblasts  
c) growing said thus transfected mouse fibroblasts in appropriated culture media, and  
d) purifying the lymphokine protein from said culture media.

35

*add  
A.B.* *John*

Fig. 1a

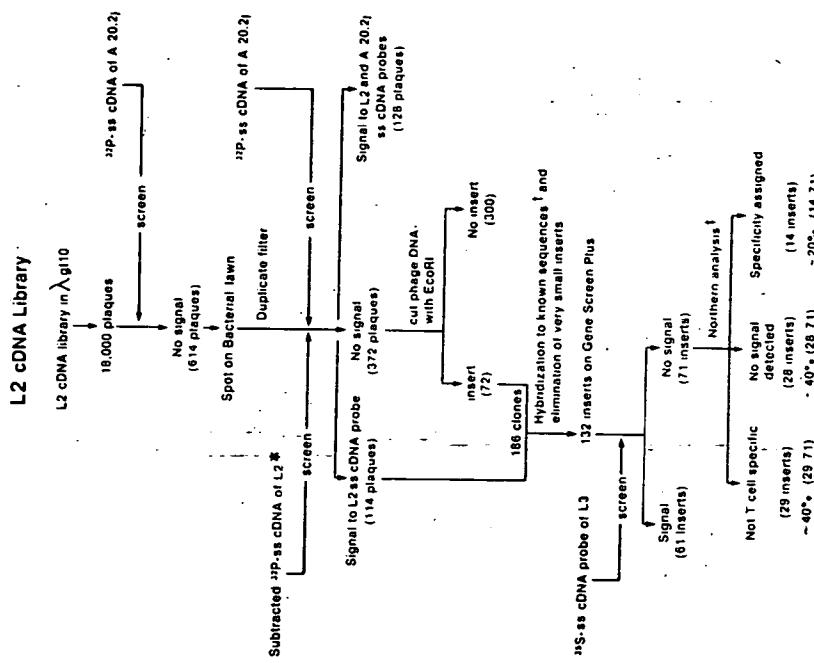
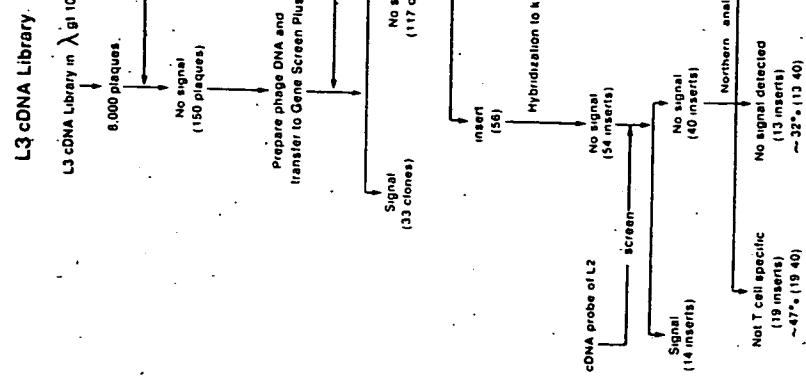


Fig. 1b



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Print of Drawing  
As Original File

Fig. 2A

Figure  
A

-47 TTTCTG TTCTGCTGAC AACGTCACCC TCTGTACCT GCTAACATC -1  
  
1 ATG AAG GTC TCC ACC ACT CCC CTT GCT GTT CTT CTC TGT ACC ATG ACA CTC TCC AAC CAA 60  
1 Met Lys Val Ser Thr Thr Ala Leu Ala Val Leu Leu Cys Thr Met Thr Leu Cys Asn Gln 20  
  
61 GTC TTC TCA CGG CCA TAT GGA CCT GAC ACC CCG ACT GCC TGC TGC TTC TCC TAC AGC CGG 120  
21 Val Phe Ser Ala Pro Tyr Gly Ala Asp Thr Pro Thr Ala Cys Cys Phe Ser Tyr Ser Arg 40  
  
121 AAG ATT CCA CGG CAA TTC ATC GTT GAC TAT TTT GAA ACC AGC AGC CTT TGC TCC CAG CCA 180  
41 Lys Ile Pro Arg Gln Phe Ile Val Asp Tyr Phe Glu Thr Ser Ser Leu Cys Ser Gln Pro 60  
  
181 GGT GTC ATT TTC CTG ACT AAG AGA AAC CCG CAG ATC TGC GCT GAC TCC AAA GAG ACC TGG 240  
61 Gly Val Ile Phe Leu Thr Lys Arg Asn Arg Gln Ile Cys Ala Asp Ser Lys Glu Thr Trp 80  
  
241 GTC CAA GAA TAC ATC ACT GAC CTG GAA CTG AAT GCC TGA GAG TCT TGG AGC CAG CGA GGA 300  
81 Val Gln Glu Tyr Ile Thr Asp Leu Glu Leu Asn Ala ---  
  
301 ACC CCC CAA ACC TCC ATG GGT CCC GTG TAG AGC AGG GGC TTG AGC CCC GGA ACA TTC CTG 350  
361 CCA CCT GCA TAG CTC CAT CTC CTA TAA GCT GTT TGC TCC CAA GTC GCC ACA TCG AGG GAC 420  
421 TCT TCA CTT GAA ATT TTA TTT AAT TTA ATC CTA TTG GTT TAA TAC TAT TTA ATT TTG TAA 480  
481 TTT ATT TTA TTG TCA TAC TTG TAT TTG TGA CTA TTT ATT CTG AAA GAC TIC AGG ACA CGT 540  
541 TCC TCA ACC CCC ATC TCC CTC CCA GTT GGT CAC ACT GTT TGG TGA CAG CTA TTC TAG GTA 600  
601 GAC ATG ATG ACA AAG TCA TGA ACT GAC AAA TGT ACA ATA GAT GCT TTG TTT ATA CCA GAG 660  
661 AAG TAA TAA ATA TGC CCT TTA ACA ACT GAA AAA AAA

Fig. 2B

B  
L2G25B (1) Met Lys Val Ser Thr Thr Ala Leu Ala Val Leu Leu Cys Thr Met Thr Leu Cys Asn Gln  
PLD78 (1) Met Gln Val Ser Thr Ala Ala Leu Ala Val Leu Leu Cys Thr Met Ala Leu Cys Asn Gln  
  
Val Phe Ser Ala Pro Tyr Gly Ala Asp Thr Pro Thr Ala Cys Cys Phe Ser Tyr - Ser  
- Phe Ser Ala Ser Leu Ala Ala Asp Thr Pro Thr Ala Cys Cys Phe Ser Tyr Thr Ser  
Arg Lys Ile Pro Arg Gln - Phe Ile Val Asp Tyr Phe Glu Thr Ser Ser Leu Cys Ser  
Arg Gln Ile Pro - Gln Asn Phe Ile Ala Asp Tyr Phe Glu Thr Ser Ser Gln Cys Ser  
Gln Pro Gly Val Ile Phe Leu Thr Lys Arg Asn Arg Gln Ile Cys Ala Asp - Ser Lys  
Lys Pro Gly Val Ile Phe Leu Thr Lys Arg Ser Arg Gln Val Cys Ala Asp Pro Ser -  
Glu Thr Trp Val Gln Glu Tyr Ile Thr Asp Ile Glu Ile Asn Ala (stop) (92)  
Glu Glu Trp Val Gln Lys Tyr Val Ser Asp Leu Glu Leu Ser Ala (stop) (92)

KWON000031

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Fig. 3A

ATGTC

-145  
-140 CATGAAGTGC TGAGTGGATA AACAGCACCG GATATCTCG TCTAAACGAA TATTACTACA CCACGGAAAG  
-70 GACACATTCG ACAACAGGAA AGGAGCCTGT CACAGAAAAC CACAGTGCC TGTCATGTG ACATTTCCCC  
1 ATG GGA AAC AAC TGT TAC AAC GTG GTG CTC ATT GTG CTG CTG CTA GTG GGC TGT GAG AAG 60  
1 Met Gly Asn Asn Cys Tyr Asn Val Val Ile Val Leu Leu Val Gly Cys Glu Lys 20  
61 GTG GGA GCC GTG CAG AAC TCC TGT GAT AAC TGT CAG CCT GGT ACT TTC TGC AGA AAA TAC 120  
21 Val Gly Ala Val Gln Asn Ser Cys Asp Asn Cys Gln Pro Gly Thr Phe Cys Arg Lys Tyr 40  
121 AAT CCA GTC TGC AAG AGC TGC CCT CCA ACT ACC TTC TCC ACC ATA CGT GGA CAG CCC AAC 180  
41 Asn Pro Val Cys Lys Ser Cys Pro Pro Ser Thr Phe Ser Ser Ile Gly Gln Pro Asn 60  
181 TGT AAC ATC TGC AGA GTG TGT GCA CGC TAT TTC AGG TTC AAG AAG TTT TGC TCC TCT ACC 240  
61 Cys Asn Ile Cys Arg Val Cys Ala Gly Tyr Phe Arg Phe Lys Phe Cys Ser Ser Thr 80  
241 CAC AAC GCG GAG TGT GAG TGC ATT GAA GGA TTC CAT TGC TTG GGG CCA CAG TGC ACC AGA 300  
81 His Asn Ala Glu Cys Glu Cys Ile Glu Gly Phe His Cys Leu Gly Pro Gln Cys Thr Arg 100  
301 TGT GAA AAG GAC TGC AGG CCT GGC CAG CAG CTA ACG AAG CAG GGT TGC AAA ACC TGT AGC 360  
101 Cys Glu Lys Asp Cys Arg Pro Gly Gln Glu Leu Thr Lys Gln Gly Cys Lys Thr Cys Ser 120  
361 TTG GGA ACA TTT AAT GAC CAG-AAC GGT ACT GGC GTC TGT CGA CCC TGG AGC AAC TGC TCT 420  
121 Leu Gly Thr Phe Asn Asp Gln Asn Gly Thr Gly Val Cys Arg Pro Trp Thr Asn Cys Ser 160  
421 CTA GAC GGA-AGC TCT GTG CTT AAC ACC GGG ACC ACC GAG GAC GAC GTG GTG TGT GGA CCC 480  
141 Leu Asp Gly Arg Ser Val Leu Lys Thr Gly Thr Thr Gly Val Lys Asp Val Val Cys Gly Pro 160  
481 CCT GTG GTG AGC TTC TCT CCC AGT ACC ACC ATT TCT GTG ACT CCA GAG GGA CCA GGA 540  
161 Pro Val Val Ser Phe Ser Pro Ser Thr Thr Ile Ser Val Thr Pro Glu Gly Gly-Pro Gly -180  
541 GGG CAC TCC TTG CAG GTC CTT ACC TTG TTC CTG GCG CTG ACA TCG GCT TTG CTG CTG GCC 600  
181 Gly His Ser Leu Gln Val Leu Thr Leu Phe Leu Ala-Leu Thr Ser Ala Leu Leu-Ala 200  
-601 CTG ATC TTC ATT ACT CTC CTG TTC TCT GTG CTC AAA TGG ATC AGG AAA AAA TTC CCC CAC 660  
201 Leu-Ile Phe Ile Thr Leu Leu Phe Ser Val Leu Lys Trp Ile Arg Lys Lys Phe Pro His 220  
661 ATA TTC AAG CAA CCA TTT AAG AAG ACC ACT GGA GCA CCT CAA-GAG CAA-GAT-GCT-TGT-AGC 720  
221 Ile Phe Lys Gln Pro Phe Lys Lys Thr Thr Gly Ala Ala Gln Glu Glu Asp Ala Cys Ser 240  
721 TGC CGA TGT CCA CAG GAA GAA GGA GGA GGA GGA GGC TAT GAG CTG TGA TGTACTATC 780  
241 Cys Arg Cys Pro Gln Glu Glu Gly Gly Gly Gly Tyr Glu Leu ---

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Fig. 3B

781 CTAGGAGATG TGCGGGCGA AACCGAGAAAG CACTAGGACC CCACCATCCT GTGGAACAGC ACAAGCAACC 850  
851 CCACCAACCT GTTCTTACAC ATCATCCTAG ATGATGTGTC GCGGGCCACC TCATCCAAGT CTCTTCTAAC 920  
921 GCTAACATAT TTGTTTAACT TCTTTTTAAAT AATTAAATT TTATGTTGT GACTGTTTG 990  
991 CCTGCCTGTA TGCACACGTG TGTGTCGTG TGTGTCGTAC ACTCCTGATG CCTGAGGAGG TCAGAACAGA 1060  
1061 AAGGGTTGGT TCCATAAGAA CTGGACTTAT GGATGGCTGT GAGCCGnnn CATAGCTGG GACGGAGACC 1130  
1131 TGTCTCTTA TTTAACGTG ACTGTATAAT AAAAAGAAAAA TGATATTCG GGAATTGTAG AGATTGTCT 1200  
1201 GACACCCCTC TAGTTAATGA TCTAACAGGA ATTGTTGATA CGTAGTATAC TGTATATGTG TATGTATATG 1270  
1271 TATATGTATA TATAAGACTC TTTACTGTC AAAGTCACC TAGAGTGTCT CGTTACCAAGG TCAATTTAT 1340  
1341 TGGACATTTT ACCTCACACA CACACACACA CACACACACA CACCTTTATA CTACGTACTGT TATCGGTAT 1410  
1411 TCTACCTCAT ATAATGGGAT AGGGTAAAGG CAAACCAAG AGTGAATGTG ATTATGTTGGA GCTGACAGA 1480  
1481 CTACCCCTTC TGGGTACCTA GGGACAGACCC TCCCTCGGAC TGTCTAAAC TCCCCTTAGA AGTCTCGTCA 1550  
1551 AGTCCCCGA CGAACAGAAC AGAGGAGACA CACTCCAAA ACTTATTTT CCGGCAAATC CTTCCCTGT 1620  
1621 TTCTGACAC TCCACCCCTT GTGGACACTT GAGTGTATC CTTGGGGGGG AAGGTCAGGT CGTACCCGTC 1690  
1691 TGTAAGGGCGG GGGAGACAGA GCGGGGGGGG AGCTACGAGA ATCCACTCAC AGGGCCGCC GGGCTCGCA 1760  
1761 AATGAAACTT TTTAAATCTC ACAAGTTTCG TCCGGCTCG GCGAACCTAT GGGCTCGATC CTIATTACCT 1830  
1831 TATCCCTGGGG CCAACATAAA ACAACAAAAA GCCTGACTC CGGTACTAAAT TCTCCCTGCC GGGCCCCGTA 1900  
1901 AGCATAACGC CCCGATCTCC ACTTTAACAA CCTGGGGGGG TTCTGGCTGG TCTGGCTTC GAAACGGTT 1970  
1971 CTTACAAAG TAATTAGTTC TTGCTTCAG CGTCAAGCT TCTGCTAGTC TATGGCAGCA TCAAGGCTG 2040  
2041 TATTGCTAC GGCTGACCCG TACCCGGCG CAATAAGGGT ACTGGGGGGC CGCTCGAAGG CCCTTGGTT 2110  
2111 TCAGAAACCC AAGGCCCCCC TCATACCAAC GTTTCGACTT TGATTCTGCC CGGTACGTTG TGGTGGTGC 2180  
2181 CTTAGCTCTT TCTCGATAGT TAG AC

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Fig. 4a

K46  
EL4 TPA  
L3  
L3 ConA  
LGL

28S-  
L3 G25#4  
18S-

Fig. 4b

K46  
EL4 TPA  
L3  
L3 ConA  
LGL

28S-  
L3 G14#2  
18S-

Fig. 4c

K46  
EL4 TPA  
L3  
L3 ConA  
LGL

L3 G20#3

28S-  
18S-

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Fig. 5

1 2 3 4



-21.7

-5.2

-2.0

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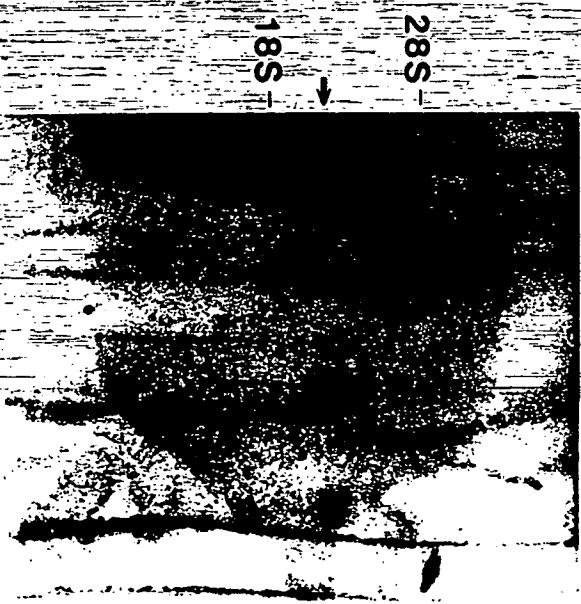
267577



a

K46  
EL4  
EL4 TPA  
L2  
L2 ConA  
L3  
L3 ConA  
LGL

Fig. 6a



b

K46  
EL4  
EL4 TPA  
L2  
L2 ConA  
L3  
L3 ConA  
LGL

Fig. 6b

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Print of Drawing  
As Original Filed

Fig. 7a

Fig. 7b

Fig. 7c

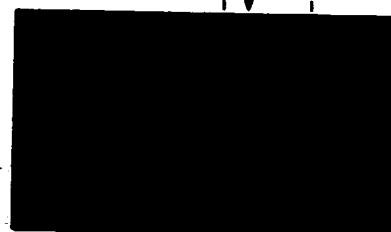
Fig. 7d

Mic after  
Slim. 0 1/2 6 12 24 6

Mic after  
Slim. 0 1/2 6 12 24 6

Mic after  
Slim. 0 1/2 6 12 24 6

Mic after  
Slim. 0 1/2 6 12 24 6



28S-  
18S-

28S-  
18S-

28S-  
18S-



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Print Of Drawing  
As Original File

a

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Fig. 3a

-8S-

-S-



b

Fig.  
60  
3b

-28S-

-18S-

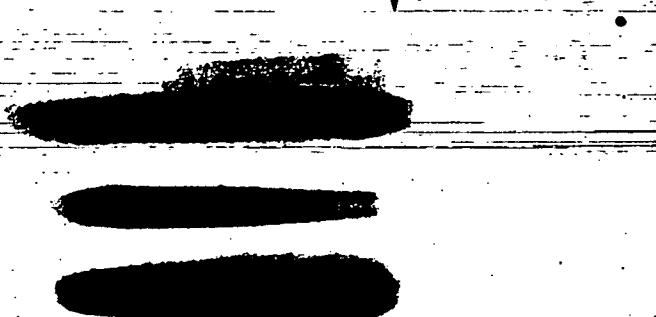


c

Fig.  
60  
3c

-28S-

-18S-



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18S-  
28S-

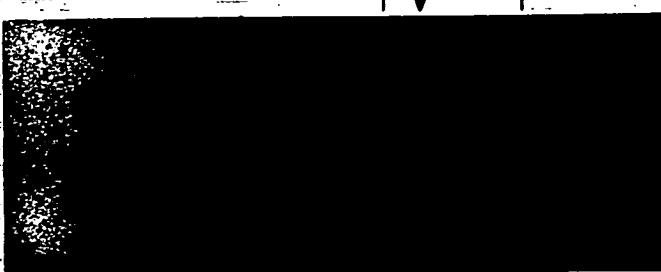


a

BW5147  
BW5147 ConA  
Md90  
Md90 ConA  
PN37  
PN37 ConA

Fig. 9a

18S-  
28S+



b

L2  
A11  
L3  
Melanocyte

Fig.  
9b

KWON000039

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K46  
EL4  
L3  
L3 ConA  
L3 ConA + CSA  
L3 ConA + ActD

Fig. 10a

188 - 288 -

b

K46  
EL4  
L3  
L3 ConA  
L3 ConA + CSA  
L3 ConA + ActD

Fig. 10b

K46  
EL4  
L3  
L3 ConA  
L3 ConA + CSA  
L3 ConA + ActD

Fig. 10c

89 - 88 -

d

K46  
EL4  
L3  
L3 ConA  
L3 ConA + CSA  
L3 ConA + ActD

Fig.  
10d

KWON000040

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a

Fig. 11a

1 2 3 4

28S -  
18S -

b

Fig. 11b

1 2 3 4

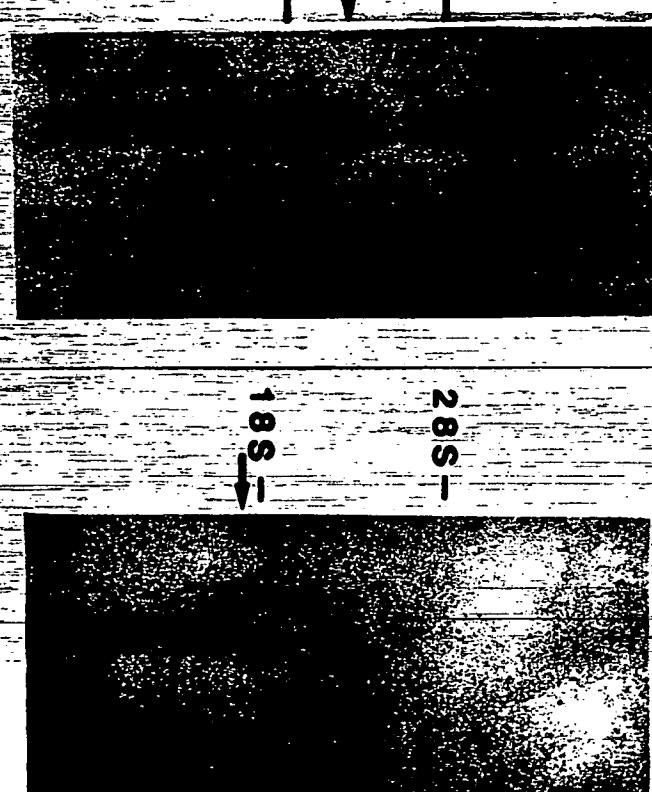
28S -  
18S -

c

Fig. 11c

1 2 3 4

28S -  
18S -



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